

MENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

Date of mailing (day/month/year) 30 January 2001 (30.01.01)	Arlington, VA 22202 ETATS-UNIS D'AMERIQUE in its capacity as elected Office
International application No. PCT/EP00/04445	Applicant's or agent's file reference B 1991 - py
International filing date (day/month/year) 16 May 2000 (16.05.00)	Priority date (day/month/year) 17 May 1999 (17.05.99)
Applicant UNSICKER, Klaus et al	

- 1. The designated Office is hereby notified of its election made:**

in the demand filed with the International Preliminary Examining Authority on:

13 December 2000 (13.12.00)

in a notice effecting later election filed with the International Bureau on:

2. The election was
 was

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

<p>The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland</p> <p>Facsimile No.: (41-22) 740.14.35</p>	<p>Authorized officer R. E. Stoffel</p> <p>Telephone No.: (41-22) 338.83.38</p>
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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70) / - /

Applicant's or agent's file reference B 1991	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/EP00/04445	International filing date (day/month/year) 16/05/2000	Priority date (day/month/year) 17/05/1999
International Patent Classification (IPC) or national classification and IPC C12N15/19		
Applicant BIOPHARM GESELLSCHAFT ZUR ... et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 9 sheets, including this cover sheet.

- This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

- I Basis of the report
- II Priority
- III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV Lack of unity of invention
- V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI Certain documents cited
- VII Certain defects in the international application
- VIII Certain observations on the international application

Date of submission of the demand 13/12/2000	Date of completion of this report 17.08.2001
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Roscoe, R Telephone No. +49 89 2399 2554



**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/EP00/04445

I. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

Description, pages:

1-17 as originally filed

Claims, No.:

1-22 as received on 25/05/2001 with letter of 23/05/2001

Drawings, sheets:

1/10-10/10 as originally filed

Sequence listing part of the description, pages:

1-5, filed with the letter of 18.08.00

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- the language of publication of the international application (under Rule 48.3(b)).
- the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- contained in the international application in written form.
- filed together with the international application in computer readable form.
- furnished subsequently to this Authority in written form.
- furnished subsequently to this Authority in computer readable form.
- The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/EP00/04445

- the description, pages:
- the claims, Nos.:
- the drawings, sheets:

5. This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):
(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

II. Priority

1. This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:
 - copy of the earlier application whose priority has been claimed.
 - translation of the earlier application whose priority has been claimed.

2. This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:
 - the entire international application.
 - claims Nos. 1-22(all part).

because:

- the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):
- the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 1-22(all part) are so unclear that no meaningful opinion could be formed (*specify*):
see separate sheet

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/EP00/04445

- the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.
 - no international search report has been established for the said claims Nos. 1-22(all part).
2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:
- the written form has not been furnished or does not comply with the standard.
 - the computer readable form has not been furnished or does not comply with the standard.

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:
 - restricted the claims.
 - paid additional fees.
 - paid additional fees under protest.
 - neither restricted nor paid additional fees.
2. This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.
3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is
 - complied with.
 - not complied with for the following reasons:
4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:
 - all parts.
 - the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes: Claims 12-14
	No: Claims 1-11, 15-22
Inventive step (IS)	Yes: Claims

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/EP00/04445

No: Claims 1-22

Industrial applicability (IA) Yes: Claims 1-22
No: Claims

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/EP00/04445

I. Basis

The documents mentioned in the present written opinion / International Preliminary Examination Report are numbered as in the search report, i.e. D1 corresponds to the first document of the search report etc.

II. Priority

The first priority date (17.05.99) is valid for the present claims. The claims of the present application are identical to the claims filed on said priority date.

III. No Opinion

No search was conducted for claims 1-22 (all part) since these relate to agonists / antagonists which are not technically defined (also no such compounds are defined in the application). Such claims are too unclear to be searched or examined.

IV. Unity

No specific objection is raised at this stage for practical reasons. It is however noted that any novel and inventive claims relating to treatment of a variety of different diseases will only be considered unitary if said diseases are linked by a novel and inventive unifying concept.

V. Reasoned statement on Novelty, Inventive Step and Industrial Applicability

It is noted that due to the unclear definitions of hGDF-15 used in the independent claims, the assessment of the claims as a whole has been conducted as though these claims refer clearly and specifically to hGDF-15 (rather than any TGF- β -like protein, for example). Otherwise, a reasonable assessment of the claims could not have been conducted.

- Novelty (Art.33(2) PCT)

Documents D1 and D2 involve the same author (Bootcov) and relate to MIC-1 or pCL13, respectively. These are alternative names for applicants hGDF-15. The entire pre-pro-mature proteins of D1 and D2 are 100% identical to presently claimed protein. The DNA sequences are 99.4% identical. Hence, D1 and D2 anticipate claims 1-11 (newly discovered intrinsic properties of a protein / DNA cannot render product as such novel). In D1, MIC-FLAG is produced and isolated, in D2 a variety of production methods are suggested (p.7). D2 further discloses antibodies against pCL13 (p.4) and pharmaceutical compositions (p.6). In addition, D2 further discloses diagnostic kits based on pCL13 (see claims 38 and 39 of D2, also p.6, l.14-16). Hence, claims 15-22 are not novel.

D3 discloses GF-2H, again an alternative term for hGDF-15. The mature protein sequences are identical, a single aa difference is found in the pre-protein. DNA sequences of pre-pro-mature encoding region 99.5% identical. D3 discloses antiserum, pharmaceutical compositions and methods of production. Anticipates claims 1-11.

It is noted that a number of further uncited documents also disclose hGDF-15. For example, WO 96/18730, JP07258293 and JP07250688.

- **Inventive Step (Art.33(3) PCT)**

D2 already suggests using hGDF-15 to treat conditions which are beneficially treatable with TGF- β or another member of superfamily (p.4, l.27). Hence, it is obvious that one could use more than one agent in combination (see claim 36 of D2). Thus, claims 12-14 are not inventive. The fact that D2 does not explicitly suggest combining medicaments is considered irrelevant since it is common medical practice to administer combination treatments. Only if a specific treatment with a surprising effect (e.g. synergistic effect) is claimed, could such a combination possibly be considered inventive.

The following indications are given in case applicant wishes to formulate medical use claims (or the European equivalent) in a regional phase. In such claims the purpose can be considered as a clear limiting feature.

Applicant primarily refers to treatment of neurodegenerative disorders in the claims. Insofar as treatments would be defined as being directed specifically to neurodegenerative disorders it may be possible to acknowledge inventive step. However, claims 9, 10 and 11 define the disorders in a broader manner. Claims 9-11 encompass compositions for treatment of brain tumors and infections of the CNS. D2 already claims use of hGDF-15 against cancers in general (see e.g. claim 37) and hence selection of brain-tumor treatment not considered inventive. Also suggests treatment of inflammatory diseases (see claim 38), which would encompass meningitus (e.g.). Hence, treatment of conditions as referred to in claims 9-11 would be considered to comprise non-inventive subject matter. It is further noted that role in tissue regeneration after wounding suggested in WO96/18730 - wounding to brain aspect would be relevant to novelty of same claims as mentioned above.

- **Industrial Applicability (Art.33(4) PCT)**

The present claims appear to have industrial applicability.

VIII. Certain observations

- **Clarity (Art.6 PCT)**

Claim 1 does not technically define the protein of the invention in an acceptable manner. Further, claim 1 is an unacceptable omnibus-style claim. Argumentation that claim is limited by structural feature is not convincing, since the feature in question "containing the 7 cys-knot region of the TGF-beta superfamily or a functionally active derivative or part thereof having at least a neurotrophic effect on DAergic neurons" is effectively no more than a functional feature. This is because an undefined derivative / part need not retain any recognizable structural feature. It is noted that no broad sequence definitions (e.g. % identities) are specified in the application as filed. Hence, the exact sequence will need to be introduced.

Since nucleic acid of claim 1 defined in an open-ended manner, protein encoded

thereby can be any protein. This problem also extends to further matter: e.g. antibody defined via the protein (antibodies must anyhow be defined via binding to an exact sequence because if variation allowed can create any small epitope for which antibodies are already known).

Further, claim wording "for the prevention and/or treatment of neurodegenerative disorders in mammals" is read as meaning suitable for such treatment. Prior art compositions intrinsically suitable are thus not excluded. Only medical treatment claims (or their equivalent) would overcome this problem.

Claim 7 "enconded", claim 12 "fuctionally"

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference B 1991 - py	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/EP 00/04445	International filing date (day/month/year) 16/05/2000	(Earliest) Priority Date (day/month/year) 17/05/1999
Applicant BIOPHARM GESELLSCHAFT ZUR BIOTECHNOLOGISCHEN ...		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

- the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
- b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing:
- contained in the international application in written form.
 - filed together with the international application in computer readable form.
 - furnished subsequently to this Authority in written form.
 - furnished subsequently to this Authority in computer readable form.
 - the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
 - the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. Certain claims were found unsearchable (See Box I).

3. Unity of invention is lacking (see Box II).

4. With regard to the title,

- the text is approved as submitted by the applicant.
- the text has been established by this Authority to read as follows:

5. With regard to the abstract,

- the text is approved as submitted by the applicant.
- the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is Figure No.

- as suggested by the applicant.
- because the applicant failed to suggest a figure.
- because this figure better characterizes the invention.

1

Non of the figures.



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

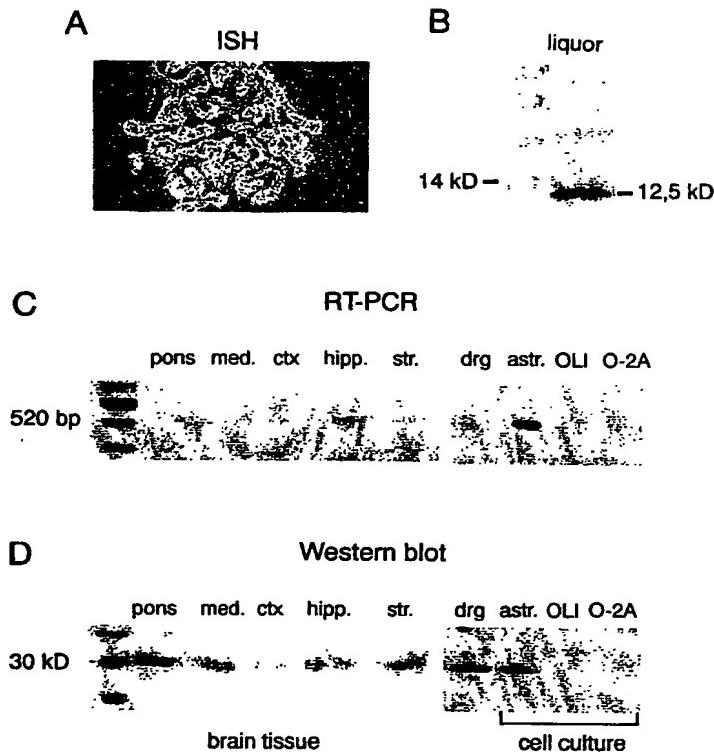
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(30) Priority Data: 99109714.8 17 May 1999 (17.05.99) EP 99114853.7 29 July 1999 (29.07.99) EP	
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(74) Agent: PERREY, Ralf; Müller-Boré & Partner, Grafinger Strasse 2, D-81671 München (DE).	

(54) Title: NEUROPROTECTIVE PROPERTIES OF GDF-15, A NOVEL MEMBER OF THE TGF- β SUPERFAMILY

(57) Abstract

The present invention relates to a transforming growth factor-beta (TGF- β)-like protein which is derived from neurons and glial cells, and which has a neurotrophic effect on dopaminergic (DAergic) neurons, to nucleic acids coding for the protein, to a vector containing the nucleic acids, to host organisms containing the nucleic acids or the vector, to antibodies directed against the protein, to methods for the production of the nucleic acids, the vector or the protein, to a pharmaceutical composition for the treatment of neurodegenerative disorders in mammals and to a diagnostic kit for the detection of said disorders.



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REPLACED BY
ART 34 AMDT

Claims

1. A nucleic acid containing a nucleotide sequence encoding the primary amino acid sequence of a TGF- β -like protein or a functionally active derivative or part thereof which is derived from neurons and glial cells and which has a neurotrophic effect on DAergic neurons.
5
2. The nucleic acid of claim 1, consisting substantially of deoxyribonucleotides and/or ribonucleotides and/or modified nucleotides.
3. The nucleic acid of claim 1 or 2, wherein the neuron and glial cells are of mammalian origin.
10
4. The nucleic acid according to anyone of claims 1 to 3, wherein the protein protects against neurodegenerative events.
- 15 5. The nucleic acid of claim 4, wherein the neurodegenerative event is mediated by oxidative damage and/or free radical damage and/or mediators and/or executors of neuronal death programs.
- 20 6. The nucleic acid according to claim 5, wherein the mediators of the free radical damage are selected from the group consisting of iron, NO donors, and other free radical donors, and the mediators and executors of neuronal death programs are selected from the group consisting of caspases and pro- and anti-apoptotic members of the bcl-2 family.

7. The nucleic acid according to anyone of claims 1 to 6, comprising at least the nucleotide sequence shown in Fig. 7A or the nucleotide sequence shown in Fig. 8A or nucleotides 40 to 333 of the nucleotide sequence shown in Fig. 8A or mutants thereof leading to the expression of functionally active polypeptides.
8. A vector containing at least the nucleic acid according to anyone of claims 1 to 7.
- 10 9. A host organism containing at least the nucleic acid according to anyone of claims 1 to 7 or the vector of claim 8.
10. A protein encoded by the nucleic acid according to anyone of claims 1 to 7.
- 15 11. An antibody or a functional fragment thereof directed against the protein of claim 10.
12. An antagonist directed to the protein of claim 10.
- 20 13. An agonist as a substitute for the protein of claim 10.
14. A method for the production of the nucleic acid according to anyone of claims 1 to 7 or the vector of claim 8 or the protein of claim 10, comprising the steps of:
 - (a) cultivating the host organism of claim 9 in a suitable medium under suitable conditions; and
 - (b) isolating the desired product from the medium and/or the host organisms.
- 30 15. A pharmaceutical composition comprising the nucleic acid according to anyone of claims 1 to 7 or the vector of claim 8 or the protein of claim 10 or the antibody of claim 11 or the antagonist of claim 12 or the agonist of

claim 13, optionally in combination with a pharmaceutically acceptable carrier and/or diluent.

16. The pharmaceutical composition of claim 15 for the prevention and/or
5 treatment of neurodegenerative disorders in mammals.

17. The pharmaceutical composition of claim 16, wherein the mammal is a
human.

10 18. The pharmaceutical composition of claim 16 or 17, wherein the neurodege-
nerative disorders are selected from the group of acute and/or chronic
neurological and psychological disorders.

15 19. The pharmaceutical composition of claim 18, wherein the neurological and
psychological disorders are caused by stroke, parkinson's disease, Alz-
heimer's disease or other dementias, infections of the CNS and psychiatric
disorders associated with disturbances in CNS transmitter systems.

20 20. The pharmaceutical composition of claim 19, wherein the psychiatric
disorders are selected from the group consisting of depression and schi-
zophrenia.

25 21. The pharmaceutical composition according to anyone of claims 15 to 20
further comprising one or more agents having neurotrophic activity or
functionally active derivatives or parts thereof.

22. The pharmaceutical composition of claim 21, wherein the agent is a cytoki-
ne.

30 23. The pharmaceutical composition of claim 22, wherein the cytokine is
selected from the group consisting of GDF, GDNF, TGF, activin A, BMP,
BDNF, NGF, EGF, CNTF and FGF.

24. A diagnostic kit comprising the nucleic acid according to anyone of claims 1 to 7 and/or the vector of claim 8 and/or the protein of claim 10 and/or the antibody of claim 11 for the detection of neurodegenerative disorders and/or infections of the CNS in mammals.

5

25. The diagnostic kit of claim 24, wherein the infection is a meningitis.

26. The diagnostic kit of claim 24 or 25, wherein the mammal is a human.

**AMENDED CLAIMS PER INTERNATIONAL PRELIMINARY EXAMINATION REPORT
DATED 17 AUGUST 2001**

Claims

1. A pharmaceutical composition comprising a nucleic acid containing a nucleotide sequence encoding the primary amino acid sequence of a protein containing the 7 Cys-knot region of the TGF- β superfamily or a functionally active derivative or part thereof having at least a neurotrophic effect on DAergic neurons, wherein the gene coding for the protein containing the 7 Cys-knot region of the TGF- β superfamily is transcribed and/or translated in neurons and glial cells, or a vector containing at least the nucleic acid or a protein encoded by the nucleic acid or an antibody or a functional fragment thereof directed against the protein or an antagonist directed to the protein or an agonist as a substitute for the protein, optionally in combination with a pharmaceutically acceptable carrier and/or diluent, for the prevention and/or treatment of neurodegenerative disorders in mammals.
2. The pharmaceutical composition according to claim 1, wherein the neuron and glial cells are of mammalian origin.
3. The pharmaceutical composition according to claim 1 or 2, wherein the protein containing the 7 Cys-knot region of the TGF- β superfamily protects against neurodegenerative events.
4. The pharmaceutical composition according to claim 3, wherein the neurodegenerative event is mediated by oxidative damage and/or free radical damage and/or mediators and/or executors of neuronal death programs.
5. The pharmaceutical composition according to claim 4, wherein the mediators of the free radical damage are selected from the

**AMENDED CLAIMS PER INTERNATIONAL PRELIMINARY EXAMINATION REPORT
DATED 17 AUGUST 2001**

group consisting of iron, NO donors, and other free radical donors, and the mediators and executors of neuronal death programs are selected from the group consisting of caspases and pro- and anti-apoptotic members of the bcl-2 family.

6. The pharmaceutical composition according to any one of claims 1 to 5, wherein the nucleic acid comprises at least the nucleotide sequence shown in Fig. 7A or the nucleotide sequence shown in Fig. 8A or nucleotides 40 to 333 of the nucleotide sequence shown in Fig. 8A or mutants thereof leading to the expression of functionally active polypeptides.
7. The pharmaceutical composition according to any one of claims 1 to 5, wherein the protein encoded by the nucleic acid comprises at least the primary amino acid sequence shown in Fig. 7B or the primary amino acid sequence shown in Fig. 8B or amino acid residues 14 to 111 of the sequence shown in Fig. 8B as well as homologs thereof having conservative amino acid substitutions.
8. The pharmaceutical composition according to any one of claims 1 to 7, wherein the mammal is a human.
9. The pharmaceutical composition according to any one of claims 1 to 8, wherein the neurodegenerative disorders are selected from the group of acute and/or chronic neurological and psychological disorders.
10. The pharmaceutical composition of claim 9, wherein the neurological and psychological disorders are caused by stroke, parkinson's disease, Alzheimer's disease or other dementias, infections of the CNS and psychiatric disorders associated with disturbances in CNS transmitter systems.

**AMENDED CLAIMS PER INTERNATIONAL PRELIMINARY EXAMINATION REPORT
DATED 17 AUGUST 2001**

11. The pharmaceutical composition according to claim 10, wherein the psychiatric disorders are selected from the group consisting of depression and schizophrenia.
12. The pharmaceutical composition according to any one of claims 1 to 11 further comprising one or more agents having neurotrophic activity or functionally active derivatives or parts thereof.
13. The pharmaceutical composition according to claim 12, wherein the agent is a cytokine.
14. The pharmaceutical composition according to claim 13, wherein the cytokine is selected from the group consisting of GDF, GDNF, TGF, activins, BMP, BDNF, NGF, EGF, CNTF and FGF.
15. A diagnostic kit comprising a nucleic acid containing a nucleotide sequence encoding the primary amino acid sequence of a protein containing the 7 Cys-knot region of the TGF- β superfamily or a functionally active derivative or part thereof having at least a neurotrophic effect on DAergic neurons, wherein the gene coding for the protein containing the 7 Cys-knot region of the TGF- β superfamily is transcribed and/or translated in neurons and glial cells, and/or a vector containing at least the nucleic acid and/or a protein encoded by the nucleic acid and/or an antibody or a functional fragment thereof directed against the protein, for the detection of neurodegenerative disorders in mammals.
16. The diagnostic kit according to claim 15, wherein the neuron and glial cells are of mammalian origin.
17. The diagnostic kit according to claim 15 or 16, wherein the protein containing the 7 Cys-knot region of the TGF- β

AMENDED CLAIMS PER INTERNATIONAL PRELIMINARY EXAMINATION REPORT
DATED 17 AUGUST 2001

superfamily protects against neurodegenerative events.

18. The diagnostic kit according to claim 17, wherein the neurodegenerative event is mediated by oxidative damage and/or free radical damage and/or mediators and/or executors of neuronal death programs.
19. The diagnostic kit according to claim 18, wherein the mediators of the free radical damage are selected from the group consisting of iron, NO donors, and other free radical donors, and the mediators and executors of neuronal death programs are selected from the group consisting of caspases and pro- and anti-apoptotic members of the bcl-2 family.
20. The diagnostic kit according to any one of claims 15 to 19, wherein the nucleic acid comprises at least the nucleotide sequence shown in Fig. 7A or the nucleotide sequence shown in Fig. 8A or nucleotides 40 to 333 of the nucleotide sequence shown in Fig. 8A or mutants thereof leading to the expression of functionally active polypeptides.
21. The diagnostic kit according to any one of claims 15 to 20, wherein the protein encoded by the nucleic acid comprises at least the primary amino acid sequence shown in Fig. 7B or the primary amino acid sequence shown in Fig. 8B or amino acid residues 14 to 111 of the sequence shown in Fig. 8B as well as homologs thereof having conservative amino acid substitutions.
22. The diagnostic kit according to any one of claims 19 to 21, wherein the mammal is a human.

io/pys

Description

The present invention relates to a transforming growth factor-beta (TGF- β)-like protein which is derived from neurons and glial cells, and which has a neurotrophic effect on dopaminergic (DAergic) neurons, to nucleic acids coding for the protein, to a vector containing the nucleic acids, to host organisms containing the nucleic acids or the vector, to antibodies directed against the protein, to methods for the production of the nucleic acids, the vector or the protein, to a pharmaceutical composition for the treatment of neurodegenerative disorders in mammals and to a diagnostic kit for the detection of said disorders.

Members of the TGF- β superfamily are known for their important multifunctional implications in development and maintenance, such as the organization of the body plan, regulation of cell proliferation, differentiation, and cell survival. The still expanding TGF- β superfamily includes the TGF- β isoforms 1 to 5, activins, inhibins, bone morphogenetic proteins (BMPs), growth/differentiation factors (GDFs), mullerian-inhibiting substance, *Drosophila* decapentaplegic gene complex, *Xenopus* Vg-1 gene, and a growing subfamily of glial cell line-derived growth factors (GDNFs) and related proteins. All members of the TGF- β superfamily share several homologous structures. They are synthesized as large precursor molecules containing a biologically inactive pro-domain which can be secreted as a complex with the mature carboxyterminal portion. Furthermore, the mature bioactive proteins are generated by proteolysis using a characteristic cleavage site. Most notably, the mature carboxy-terminal segments contain a highly conserved cystein knot.

As TGF- β -like proteins are also implicated in the regulation of neuronal stem cell proliferation and maintenance of neurons there is a great demand for novel members of this protein family.

Accordingly, the technical problem underlying the present invention is to provide novel compounds relating to TGF- β -like proteins having neurotrophic activities which are suitable for the treatment and diagnosis of neurodegenerative disorders.

The solution to the above technical problem is achieved by providing the embodiments as characterized in the claims.

In particular, the present invention relates to a nucleic acid containing a nucleotide sequence encoding the primary amino acid sequence of a TGF- β -like protein or a functionally active derivative or part thereof which is derived from neurons and glial cells and which has a neurotrophic effect on DAergic neurons.

The terms "nucleic acid" and "nucleotide sequence" refer to endogenously expressed, semi-synthetic, synthetic or chemically modified nucleic acid molecules, preferably consisting substantially of deoxyribonucleotides and/or ribonucleotides and/or modified nucleotides. Further, the term "nucleotide sequence" may comprise exons, wherein the nucleotide sequence encodes the primary amino acid sequence and may be degenerated based on the genetic code. The term "primary amino acid sequence" refers to the sequence of amino acids irrespective of tertiary and quaternary protein structure.

The term "TGF- β -like protein" refers to proteins displaying the characteristics of the TGF- β superfamily, especially a conserved cystein rich motif, and comprises both the large precursor molecules containing a pro-domain as well as the mature bioactive proteins which are generated by proteolysis using a characteristic cleavage site.

The terms "functionally active derivative" and "functionally active part" refer to a proteinaceous compound exhibiting at least a neurotrophic effect on DAergic neurons. The functionally active form of the above-defined TGF- β -like protein may be a monomeric, dimeric and/or oligomeric form, as well as a heterooligomeric form, e.g. a heterodimer, comprising at least two different monomers of TGF- β -like proteins having neurotrophic activity.

The expression "derived from neurons and glial cells" means that the gene coding for the protein is transcribed and/or translated in neurons and glial cells such as Purkinje cells and astrocytes such that the mRNA and/or the protein is detectable by methods known in the art such as *in situ* hybridization, RT-PCR, Northern or Western blotting.

The expression "neurotrophic effect on DAergic neurons" refers

to a proteinaceous activity that may confer, by itself or in combination with other factors, survival and differentiation upon DAergic neurons within the nanomolar range or below.

5 In a preferred embodiment of the above defined nucleic acid the neurons and glial cells are of mammalian origin, e.g. human, mouse or rat.

In a further preferred embodiment, the TGF- β -like protein protects against neurodegenerative events. Such neurodegenerative events may be e.g. mediated by oxidative damage, free radicals, 10 mediators or executors of neuronal death programs such as caspases, pro- and anti-apoptotic members of the bcl-2 family. A toxic radical damage may be mediated by iron, e.g. Fe-ions, NO and other radical donors. Therefore, the nucleic acid as defined above encodes a TGF- β -like protein which is able to protect DAergic 15 neurons against intoxication by iron, which is suggested to cause Parkinson's disease (PD).

In a further preferred embodiment the nucleic acid according to the present invention comprises at least the nucleotide sequence shown in Fig. 7A or the nucleotide sequence shown in Fig. 8A or 20 nucleotides 40 to 333 of the nucleotide sequence shown in Fig. 8A or mutants of such nucleic acids leading to the expression of functionally active polypeptides. Examples of such mutations include deletions, insertions and substitutions of one or more nucleotides such as mutations which lead to conservative amino acid 25 substitutions, e.g. such mutations in the range of nucleotides 40 to 333 of the nucleotide sequence shown in Fig. 8A, i.e. the region of the nucleotide sequence encoding the 7 Cys-knot region which is highly conserved in TGF- β -like proteins.

A further subject of the present invention relates to a vector 30 containing at least the nucleic acid as defined above. The term "vector" refers to a DNA and/or RNA replicon that can be used for the amplification and/or expression of the above defined nucleotide sequence. The vector may contain any useful control units such as promoters, enhancers, or other stretches of sequence within the 5' 35 regions of the sequence serving for the control of its expression. The vector may additionally contain sequences within the 5' and/or 3' region of the nucleotide sequence, that encode amino acid

sequences such as a His-tag which are useful for the detection and/or isolation of the protein encoded by the nucleotide sequence. Furthermore, the vector may contain sequence elements within the 5' and/or 3' region of the nucleotide sequence encoding amino acid 5 sequences which serve for the targeting of the protein encoded by the nucleotide sequence to nerve tissues and/or for the penetration of the blood/brain barrier. Examples of suitable vectors are baculovirus vectors.

Another embodiment of the present invention relates to a host 10 organism containing the nucleic acid or the vector, as defined above. The term "host organism" comprises a virus, a bacterium such as *Escherichia coli*, a fungus, a plant, a mammal or an insect or parts such as cells, e.g. Sf9 cells, thereof.

A further embodiment of the present invention relates to the 15 protein itself, which is encoded by the nucleic acid as defined above. Examples of the primary amino acid sequence of the protein according to the present invention are given in Figs. 7B and 8B, respectively. Further examples of the primary amino acid sequence of the protein according to the present invention comprise amino 20 acid residues 14 to 111 of the sequence shown in Fig. 8B as well as homologs thereof having conservative amino acid substitutions.

A further subject of the present invention relates to an antibody, which may be monoclonal or polyclonal, or a functional fragment thereof directed against the protein or a functional 25 derivative or part thereof as defined above. Further subjects of the present invention relate to an antagonist directed to the above-defined protein and to an agonist as a substitute for the above-defined protein.

A modulation of the functional activity of the above-defined 30 protein may also be achieved by altering the expression of the nucleotide sequence of the above-defined nucleic acid as compared to the expression level in a normal cell. For example, an antisense nucleic acid masking the mRNA or a ribozyme cleaving the mRNA may be used to inhibit the expression. Alternatively, the efficiency 35 of the promoter which regulates the expression of the nucleotide sequence of the above-defined nucleic acid may be influenced.

A further embodiment of the present invention relates to a

method for the production of the nucleic acid, the vector, or the protein as defined above, comprising the steps of:

- (a) cultivating the above-defined host organism in a suitable medium under suitable conditions; and
- 5 (b) isolating the desired product from the medium and/or the host organisms.

A preferred embodiment of the method for the production of the protein according to the present invention uses bacteria such as *E. coli* as the host organism. The expression of the above-defined 10 protein may then lead to a functionally inactive form, e.g. of amorphous aggregates within the bacterium known in the art as "inclusion bodies". Therefore, the method of the present invention may further comprise steps serving for the refolding and/or modification of the isolated protein into a functionally active 15 form which may be a monomeric, dimeric or oligomeric form. In particular, the present invention further comprises a method for the production of the biologically active dimeric form of the protein as defined above, preferably GDF-15, from its denatured or otherwise non-native form. This object of the present invention is 20 achieved by the unexpected finding that considerable amounts of the desired dimeric products are obtained by subjecting the monomeric form of the protein according to the present invention to refolding conditions. Thus, the present invention also relates to dimeric 25 biologically active GDF-15 which has been produced by the above-defined method.

A further embodiment of the present invention relates to a pharmaceutical composition comprising the nucleic acid or the vector or the protein or the antibody or the antagonist or the agonist as defined above, optionally in combination with a 30 pharmaceutically acceptable carrier and/or diluent. The pharmaceutical composition may be used for the prevention and/or treatment of neurodegenerative disorders in mammals, preferably in humans. Furthermore, therapeutic techniques for the treatment of disorders 35 which are associated with the expression of the nucleotide sequence of the nucleic acid according to the present invention may be designed using the above-mentioned agents which are capable of regulating the expression of the nucleotide sequence of the above-

defined nucleic acid, e.g. antisense nucleic acids, ribozymes and/or agents for influencing promoter activity. The neurodegenerative disorders are preferably acute and/or chronic neurological and psychological disorders, and may be caused by
5 stroke, parkinson's disease, Alzheimer's disease or other dementias, infections of the CNS and psychiatric disorders associated with disturbances in CNS transmitter systems such as depression and schizophrenia.

In a further preferred embodiment, the pharmaceutical
10 composition according to the present invention further comprises, in addition to the nucleic acid or the vector or the protein or the antibody or the antagonist or the agonist as defined above, one or more other agents having neurotrophic activity. Preferred agents are, e.g., cytokines or functionally active derivatives or parts
15 thereof. Preferred cytokines used in the pharmaceutical composition according to the present invention may be selected from the group consisting of GDF such as GDF-5, GDF-6, GDF-7, GDF-8 and GDF-9, GDNF, TGF such as TGF- α or TGF- β , e.g. TGF- β 1, TGF- β 2 or TGF- β 3, activin A, BMP such as BMP-2, BMP-4, BMP-6, or BMP-7, BMP-11, BMP-
20 12, BDNF, NGF, neurotrophines such as NT-3 or NT-4, EGF, CNTF and FGF such as FGF-2. The term "GDNF" includes GDNF, neurturin and persephin.

A further subject of the present invention relates to a diagnostic kit comprising the nucleic acid, the vector, the protein
25 and/or the antibody as defined above, for the detection of neurodegenerative disorders and/or infections of the CNS such as meningitis, e.g. a bacterial meningitis, in mammals, preferably humans. Examples of other neurodegenerative disorders are as defined above.
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The figures show:

Fig. 1 Localization of GDF-15 in the CNS. (A) Photographic image of an *in situ* hybridization of an adult rat choroid plexus performed with rat specific GDF-15 antisense-RNA probes. (B) Photographic image of an immunoblot analysis of human cerebrospinal fluid (CSF)
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under reducing conditions with purified GDF-15 antiserum. (C) RT-PCR of different P0 rat brain regions (pons, medulla oblongata, cortex, hippocampus, striatum), dorsal root ganglia (DRG), cultured primary astrocytes (astr.), oligodendroglial cell line OLI-neu (OLI), and cultured oligodendroglial progenitors (O-2A). (D) Immunoblot analysis under native conditions of the corresponding brain areas and cells of (c) with purified GDF-15 antiserum.

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Fig. 2 Image of Western blot analysis of GDF-15 in human CSF under reducing conditions. Molecular weight marker (St.). CSF sample of a patient with bacterial meningitis (lane 1). CSF sample of a control patient (lane 2).

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Fig. 3 Graphic representation of experiments showing the survival effect of GDF-15 in mesencephalic neuron cultures. Numbers of surviving tyrosine hydroxylase (TH)-immunoreactive neurons of mesencephalic cultures (E15/DIV7) treated with medium only (control), purified lysate from uninfected Sf9 cells (baculo control), GDF-15 (0.01 to 1 ng/ml) purified from infected Sf9 cell lysate, and GDNF (10 ng/ml). Data are given as mean ± SEM (n=3), P-values derived from Student's t-test are ***P<0.001, **P<0.01 for increased survival as compared with control cultures.

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Fig. 4 Protective effect of GDF-15 in Fe²⁺ (100 µM) treated cultures. (A) Graphic representation of numbers of surviving TH-immunoreactive neurons of mesencephalic cultures (E15/DIV7) treated with or without Fe²⁺ in medium only (control), in presence of NT-4 (10 ng/ml), and in presence of GDF-15 (10 ng/ml). (B) Graphic representation of the percentage of surviving TH-immunoreactive neurons of mesencephalic cultures (E15/DIV7) treated with Fe²⁺ in medium only (control), in

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presence of NT-4 (10 ng/ml), and in presence of GDF-15 (10 ng/ml). Values of cultures without addition of iron are set to 100%. Data are given as mean \pm SEM (n=3), P-values derived from Student's t-test are *P<0.05 for increased survival as compared with control cultures.

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Fig. 5

In vivo neurotrophic effects of GDF-15. (A) Graphic representation of amphetamine rotation data of rats with unilateral 6-OHDA (6-hydroxydopamine) lesions. Rotations per minute were monitored for 60 min beginning 5 min after amphetamine (5mg/kg i.p.) administration. (B) Graphic representation of counts of TH-positive neurons in SNpc. Values are given as percentage of TH-positive neurons of the lesioned as compared to the unlesioned side. Data are given as mean \pm SEM (n=4). P-values derived from Student's t-test are *P<0.05, **P<0.01, ***P<0.001.

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Fig. 6

Signalling of GDF-15 through Smad proteins. (A) Graphic representation of experiments demonstrating the activation of the Smad Binding Element (SBE) by TGF- β 1 and GDF-15 in transient transfected hFob cells. (B) Graphic representation of experiments showing that the PAI-1 promoter which is exclusively activated by TGF- β 1 to - β 3 in stable transfected MLEC cells does not respond to GDF-15.

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Fig. 7

(A) cDNA and (B) corresponding amino acid sequence of human pre-pro-mature GDF-15. Nucleotides and amino acids are abbreviated according to the international one letter codes.

Fig. 8 (A) cDNA and (B) corresponding amino acid sequence of human mature GDF-15.

The following non-limiting example illustrates the invention:

EXAMPLE

Identification of GDF-15.

Using the conserved cystein knot motif of TGF- β -like proteins, a combined approach employing RT-PCR and library screening revealed the full-length cDNA sequence of a novel member of the TGF- β superfamily derived from neurons. The cDNA has the sequence shown in Fig. 7A corresponding to the amino acid sequence shown in Fig. 7B.

According to a possible alternative translation start codon which is located 39 nucleotides upstream from the first nucleotide of the sequence shown in Fig. 7A, the corresponding protein may also comprise 13 additional amino acids (MPGQELRTLNGSQ) N-terminal to the sequence shown in Fig. 7B.

The protein, which is named GDF-15, was recombinantly expressed using the baculovirus system. Furthermore, an antibody against a specific peptide derived from the murine and rat C-terminal sequence (HRTDSGVSLQTYDDL) has been developed. Due to the high homology of the corresponding region of the human sequence (QKTDTGVLQTYDDL), this antibody recognizes also human GDF-15.

Localization of GDF-15 in the CNS

In situ hybridization with GDF-15 antisense RNA probes, RT-PCR as well as Western blot analyses were performed to study the distribution of GDF-15 in the CNS (Fig. 1). *In situ* hybridization revealed signals in neurons, especially Purkinje cells, in the cerebellum, and strong expression in the choroid plexus (Fig. 1A) of newborn and adult rats. RT-PCR and Western blotting of samples taken from different regions of newborn and adult rat brains and peripheral nervous system extended these results by detecting mRNA and protein in pons, medulla oblongata, midbrain, striatum, hippocampus, cortex, and dorsal root ganglia (Fig. 1C, D). Highest levels of mRNA expression were found in the choroid plexus (Fig. 1A). Antibodies raised against the above C-terminal peptide were used for Western blots. Analysis of samples of different brain areas of newborn rats revealed one distinct band at 31 kDa (Fig. 1D). The relative mass of cellular GDF-15 is in good agreement with

the theoretical molecular weight of 31 kDa of the pro-protein.

As GDF-15 is abundant in the choroid plexus, the presence of the protein in CSF of healthy human subjects as well as patients with different neurological disorders was also tested. In contrast

5 to the intracellular protein detected in brain samples, CSF samples revealed a single band at about 12 kDa under reducing conditions representing the secreted mature portion of GDF-15. Highest amounts of protein in CSF were seen in patients with bacterial meningitis (Fig. 2). Taken together these data provide evidence that GDF-15,
10 a novel member of the TGF- β superfamily, is widely expressed in various regions of the CNS including CSF and peripheral nervous system. Furthermore, GDF-15 is significantly increased in the CSF of patients with inflammatory neurological disease providing the opportunity to employ antibodies to GDF-15 as diagnostic tools in
15 neurological disease.

Production of dimeric, biologically active GDF-15

2 μ g of monomeric GDF-15 protein (e.g. produced in bacteria such as *E. coli*) is dissolved in 2917.7 μ l solubilisation buffer (1 M NaCl, 50 mM Tris-HCl, 50 mM EDTA, pH 9.5). To the thus dissolved protein, the following is added (resulting in a total volume of 3580 μ l):

35.8 μ l 100 mM oxidized Glutathion (GSSG)

35.8 μ l 200 mM reduced Glutathion (GSH)

25 590.7 μ l CHAPS (3-[(Cholamidopropyl)-dimethylamino]-1-propane sulfonate)

After incubation at 20 to 22°C for 48 h, more than 80%, typically 90%, of the monomeric protein is refolded into the
30 desired dimeric product. The separation of the dimer is performed by standard chromatographic methods such as reverse phase HPLC.

Functional studies using recombinant human GDF-15

Using the baculovirus system, the mature part of the human recombinant GDF-15 protein was expressed in Sf9 cells. However, the same results in all functional studies are obtained when using
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recombinant human GDF-15 expressed in bacteria which has been renatured by the above-described refolding method. Western blot showed the monomeric or dimeric form of the recombinant protein under reducing and non-reducing conditions, respectively. Following 5 purification, the protein was tested for its survival effects on rat embryonic midbrain DAergic neurons. Addition of recombinant GDF-15 to cultures of E14 midbrain cells augmented numbers of surviving tyrosine hydroxylase (TH)-positive neurons after 7 days *in vitro* compared to control cultures (Fig. 3). The dopaminotrophic 10 effect of GDF-15 is comparable to the documented survival promoting activity of other members of the TGF- β superfamily and the neurotrophin family (e.g. TGF- β , GDNF-subfamily members, or BDNF). Analysis of midbrain cultures using immunocytochemistry and antibodies to the astrocyte-specific intermediate filament protein 15 GFAP and assays for cell proliferation provided evidence that GDF-15 application did not exert its survival promoting effect through numerically increasing cells and promoting maturation of astrocytes, a well-established source of neurotrophic factors. This provides evidence that GDF-15 affects dopaminergic neurons directly 20 rather than indirectly, as shown for FGF-2 or BMPs.

In order to investigate whether GDF-15 is also able to protect DAergic neurons against a likely cause of PD, i.e. iron intoxication, its effects on iron-intoxicated mesencephalic neurons was examined (Fig. 4A, B). Exposure of cultures to iron (Fe^{2+}) 25 caused a 80% reduction in neuronal survival compared to untreated control cultures. Cell losses were reduced to 50% when cultures were co-treated with Fe^{2+} and GDF-15. These data strongly suggest that GDF-15 protects DAergic neurons against iron-mediated (oxidative) damage. The data also support the use of GDF-15 as an 30 agent to prevent or slow down neurodegenerative events mediated by free radicals, oxidative stress, mediators and executors of neuronal death programs.

Furthermore, it was established that GDF-15 also protects lesioned DAergic midbrain neurons *in vivo*. The nigrostriatal system 35 of adult rats was lesioned by an unilateral injection of 6-hydroxydopamine (6-OHDA) just above the left substantia nigra (SN). The results of these experiments are shown in Tables 1A and B,

respectively. The data shown in Table 1B are also represented graphically in Fig. 5A.

5 Table 1: Amphetamine rotation data

A: Rotations per minute for 60 min beginning 5 min after amphetamine administration (5 mg/kg i.p.)

Rat no.	Treatment	Rotations per min
1	6-OHDA	13
2	6-OHDA	11
3	6-OHDA	9
4	6-OHDA	11
5	6-OHDA + GDF-15	0
6	6-OHDA + GDF-15	1
7	6-OHDA + GDF-15	2
8	6-OHDA + GDF-15	0

B: Mean values

Treatment	Rotations per min (mean ± SD)
6-OHDA	11.0 ± 1.4
6-OHDA + GDF-15	0.8 ± 0.8

All rats displayed the typical features of amphetamine challenge, such as stereotypy and piloerection. Rats which had been treated with 6-OHDA only showed rotation rates of 11.0 ± 1.41 (mean ± SD), indicating at least 95% depletion of the nigrostriatal pathway (Ungerstedt et al. (1970), Brain. Res., 24, 485-493). In contrast, rats which were also treated with GDF-15 rotated at very low rates (0.75 ± 0.83), showing that this protein effectively prevented 6-OHDA-induced depletion of dopamine in the left striatum; cf. also Fig. 5A.

Furthermore, in order to confirm that the above prevention of 6-OHDA-induced dopamine depletion in the left striatum is due to a neuroprotective effect of GDF-15 on neurons in the SN, the SN pars compacta (SNpc) was analysed immunocytochemically. Counts of TH-positive neurons in the SNpc measured at three individual levels are shown in Table 2A and the mean values for each rat are given in Table 2B, respectively. The overall mean values for the 6-OHDA-treated (n=4) and for the rats which were co-treated with 6-OHDA and GDF-15 (n=4) are shown in Table 2C. The data shown in Table 2C are also represented graphically in Fig. 5B. The results show that the co-treatment of the rats with 6-OHDA and GDF-15 led to a 10-fold increase in the count of TH-positive neurons in the left striatum compared to the treatment with 6-OHDA alone. Therefore, GDF-15 prevents 6-OHDA-induced depletion of dopamine in the left striatum due to its strong neuroprotective effect on TH-immunoreactive neurons.

Table 2: TH-immunocytochemistry data

A: Counts of TH-positive neurones in substantia nigra pars compacta at three levels; -2.8, -3.0 and -3.2, relative to bregma (according to Pellegrino et al., A stereotaxic atlas of the rat brain. Plenum Press, New York, 1979)

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Rat no.	Treatment	TH counts (-2.8)			TH counts (-3.0)			TH counts (-3.2)		
		Rig ht	Lef t	L/R (%)	Rig ht	Lef t	L/R (%)	Rig ht	Le ft	L/R (%)
1	6-OHDA	102	6	5.9	121	8	6.6	125	11	8.8
2	6-OHDA	114	11	9.6	117	10	8.5	114	12	10.5
3	6-OHDA	98	3	3.1	104	4	3.8	106	9	8.5
4	6-OHDA	99	3	3.0	112	7	6.3	97	6	6.2
5	6-OHDA + GDF-15	107	69	64.5	111	73	65.8	114	81	71.1
6	6-OHDA + GDF-15	110	71	64.5	109	65	59.6	120	84	70.0
7	6-OHDA + GDF-15	114	78	68.4	123	101	82.1	126	97	77.0
8	6-OHDA + GDF-15	115	80	69.6	118	95	80.5	118	89	75.4

B: Mean values of individual rats

Rat no.	Treatment	TH counts (mean ± SD)		
		Right	Left	L/R (%)
1	6-OHDA	116.0±10.0	8.3±2.1	7.1±1.2
2	6-OHDA	115.0±1.4	11.0±0.8	9.5±0.8
3	6-OHDA	102.7±3.4	5.3±2.6	5.1±2.4
4	6-OHDA	102.7±6.6	5.3±1.7	5.2±1.5
5	6-OHDA + GDF-15	110.7±2.9	74.3±5.0	67.1±2.9
6	6-OHDA + GDF-15	113.0±5.0	73.3±7.9	64.7±4.2
7	6-OHDA + GDF-15	121.0±5.1	92.0±10.0	75.8±5.7
8	6-OHDA + GDF-15	117.0±1.4	88.0±6.2	75.2±4.5

C: Mean values of 6-OHDA-treated rats and mean values after co-treatment with 6-OHDA plus GDF-15

Treatment	TH counts (mean ± SD)		
	Right	Left	Left/Right (%)
6-OHDA	109.1±6.4	7.5±2.4	6.7±1.8
6-OHDA + GDF-15	115.4±3.9	81.9±8.2	70.7±4.9

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In summary, the above *in vivo* studies demonstrate that injections of GDF-15 immediately prior to 6-OHDA above the left SN and into the left lateral ventricle prevented 6-OHDA-induced pathological rotation behavior (Fig. 5A) and significantly reduced 10 losses of DAergic SN neurons (Fig. 5B). Together, these data show that GDF-15 can be profitably employed to ameliorate consequences of nigrostriatal degeneration in Parkinson's disease.

Using the plasmid Smad Binding Element (pSBE) which is activated by TGF- β 1, OP-1 (also referred to as BMP-7), activin, 15 BMP-2 and GDF-5, the further question was addressed as to whether GDF-15 is able to induce intracellular signal transduction through Smad proteins. Transient transfection of the human osteoblast cell line (hFob) with SBE showed that GDF-15 administration increased the luciferase signal (Fig. 6A). These results demonstrate that 20 GDF-15 activates the Smad responsive promoter element of the reporter gene construct. In a further experiment the inducibility of the Plasminogen Activator Inhibitor promoter (PAI) in stable transfected Mink Lung Epithelial Cells (MLEC) by GDF-15 was tested. The MLEC assay, which is exclusively sensitive for TGF- β 1, - β 2, 25 and - β 3, revealed no effect of GDF-15 (Fig. 6B). Since Smad2 and Smad3 phosphorylation is specifically associated with the TGF- β -mediated activation of TGF- β receptors, it is concluded that GDF-15

seems not to signal through the Smad2/3 pathway. With regard to the GDF-15-dependent activation of SBE, which is a response element for both, the Smad2/3, and the BMP-mediated Smad1/5 pathway, it appears that GDF-15 exerts its cellular effects by binding to BMP-like
5 receptors.

Summary

In conclusion, a novel neurotrophic molecule derived from neuron cells belonging to the TGF- β superfamily, GDF-15, was
10 discovered, cloned, expressed and functionally characterized.

In the nervous system, GDF-15 mRNA and protein can be detected, e.g. in midbrain, striatum and in cortex, but highest levels of the mRNA and the protein are found in the choroid plexus and spinal fluid (CSF), respectively. Interestingly, levels of
15 protein in CSF are increased in certain neurological disorders, e.g. in patients with bacterial meningitis. In order to elucidate its functions, the mature form of human GDF-15 was recombinantly expressed using a baculovirus expression system. Expression resulted in the synthesis of the biologically active dimeric form
20 of the protein. *In vitro* experiments using dissociated cell cultures of embryonic rat midbrain neurons revealed that GDF-15 can act as a neurotrophic factor for DAergic midbrain neurons which degenerate in Parkinson's disease (PD). GDF-15 is also able to protect these neurons against intoxication by iron, which may be
25 causal to PD. Furthermore, it could be demonstrated that GDF-15 also exhibits its neuroprotective effect *in vivo*. Concerning the signalling pathway GDF-15 acts upon, it was established that GDF-15 is able to induce intracellular signal transduction through Smad proteins.

30 Therefore, it can be concluded that GDF-15 has important functions in the developing, mature, and lesioned brain involving options to use GDF-15 for the treatment and diagnosis of acute and chronic neurological and psychological disorders, such as stroke, Alzheimer's disease and other demetias, and psychiatric disorders
35 associated with disturbances in CNS transmitter systems.

Methods for in vivo studies demonstrating the protective effect of GDF-15 on 6-OHDA-lesioned nigrostriatal neurons

Adult female Wistar rats were anaesthetised using ketamine (75 mg/kg i.p.) and xylazinum (15 mg/kg i.p.) and placed in a Kopf stereotaxic frame. GDF-15 was used at a final concentration of 2 µg/µl in 10 mM phosphate-buffered saline (PBS), pH 7.4. Four rats received injections of 20 µg GDF-15 just above the left substantia nigra (SN) and 20 µg GDF-15 into the left lateral ventricle (LV). This was followed immediately by an injection of 6-hydroxydopamine hydrobromide (8 µg as the free base in 4 µl 0.9% saline with 0.1% ascorbic acid) into the left medial forebrain bundle (MFB). Four additional rats received 6-OHDA only. Stereotaxic co-ordinates (Pellegrino et al. A stereotaxic atlas of the rat brain. Plenum Press, New York, 1979) were as follows: AP -3.0, LV +2.5, DV -8.5 for the SN; AP +1.0, LV +1.2, DV -3.5 for the LV; AP -2.2, LV +1.5, DV -7.9 for the MFB. All rats were tested behaviourally at seven days after surgery. Ipsilateral rotations were counted over a 60 min period beginning 5 min after (+)-amphetamine sulphate administration (5 mg/kg, i.p.). At ten days after surgery, all rats were terminally anaesthetised with chloroform/ether and perfused intracardially with 200 ml of cold 0.1 M phosphate-buffered saline (PBS), pH 7.4, containing 500 Units heparin, followed by 300 ml freshly prepared 4% paraformaldehyde in PBS. Brains were removed and placed in 4% paraformaldehyde in PBS overnight, cryoprotected in 30% sucrose in PBS and then frozen. Serial 30 µm coronal cryosections through the SN pars compacta (SNpc) were cut and stained immunocytochemically for tyrosine hydroxylase (TH). Sections were incubated in blocking solution (3% normal goat serum, 0.2% Triton X-100 in PBS) overnight at 4°C, then in a 1:2000 solution of rabbit antiserum to TH (Affiniti Labs, U.K.) in blocking solution overnight at 4°C. Sections were washed five times in PBS containing 0.02% Triton X-100, then incubated in a solution of 1:1000 horse radish peroxidase-linked anti-rabbit IgG (Vector Labs) overnight at 4°C. After washing as before, TH immunostaining was visualised using 3,3'-diaminobenzidine as the chromogen. Sections were mounted onto gelatinised slides, dehydrated in alcohol, cleared in xylene and mounted in DePeX® (Bioproducts,

Heidelberg, Germany). TH-immunoreactive neurons were counted in the SNpc on both sides of the brain at each of three levels; -2.8, -3.0, -3.2, with respect to bregma (Pellegrino et al., 1979).

Claims

1. A nucleic acid containing a nucleotide sequence encoding the
5 primary amino acid sequence of a TGF- β -like protein or a functionally active derivative or part thereof which is derived from neurons and glial cells and which has a neurotrophic effect on DAergic neurons.
- 10 2. The nucleic acid of claim 1, consisting substantially of deoxyribonucleotides and/or ribonucleotides and/or modified nucleotides.
- 15 3. The nucleic acid of claim 1 or 2, wherein the neuron and glial cells are of mammalian origin.
4. The nucleic acid according to anyone of claims 1 to 3, wherein the protein protects against neurodegenerative events.
- 20 5. The nucleic acid of claim 4, wherein the neurodegenerative event is mediated by oxidative damage and/or free radical damage and/or mediators and/or executors of neuronal death programs.
- 25 6. The nucleic acid according to claim 5, wherein the mediators of the free radical damage are selected from the group consisting of iron, NO donors, and other free radical donors, and the mediators and executors of neuronal death programs are selected from the group consisting of caspases and pro- and
30 anti-apoptotic members of the bcl-2 family.
7. The nucleic acid according to anyone of claims 1 to 6, comprising at least the nucleotide sequence shown in Fig. 7A or the nucleotide sequence shown in Fig. 8A or nucleotides 40 to 333 of the nucleotide sequence shown in Fig. 8A or mutants thereof leading to the expression of functionally active polypeptides.
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8. A vector containing at least the nucleic acid according to anyone of claims 1 to 7.

5 9. A host organism containing at least the nucleic acid according to anyone of claims 1 to 7 or the vector of claim 8.

10 10. A protein encoded by the nucleic acid according to anyone of claims 1 to 7.

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11. An antibody or a functional fragment thereof directed against the protein of claim 10.

12. An antagonist directed to the protein of claim 10.

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13. An agonist as a substitute for the protein of claim 10.

14. A method for the production of the nucleic acid according to anyone of claims 1 to 7 or the vector of claim 8 or the 20 protein of claim 10, comprising the steps of:

(a) cultivating the host organism of claim 9 in a suitable medium under suitable conditions; and

(b) isolating the desired product from the medium and/or the host organisms.

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15. A pharmaceutical composition comprising the nucleic acid according to anyone of claims 1 to 7 or the vector of claim 8 or the protein of claim 10 or the antibody of claim 11 or the antagonist of claim 12 or the agonist of claim 13, optionally 30 in combination with a pharmaceutically acceptable carrier and/or diluent.

16. The pharmaceutical composition of claim 15 for the prevention and/or treatment of neurodegenerative disorders in mammals.

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17. The pharmaceutical composition of claim 16, wherein the mammal is a human.

18. The pharmaceutical composition of claim 16 or 17, wherein the neurodegenerative disorders are selected from the group of acute and/or chronic neurological and psychological disorders.

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19. The pharmaceutical composition of claim 18, wherein the neurological and psychological disorders are caused by stroke, parkinson's disease, Alzheimer's disease or other dementias, infections of the CNS and psychiatric disorders associated with disturbances in CNS transmitter systems.

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20. The pharmaceutical composition of claim 19, wherein the psychiatric disorders are selected from the group consisting of depression and schizophrenia.

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21. The pharmaceutical composition according to anyone of claims 15 to 20 further comprising one or more agents having neurotrophic activity or functionally active derivatives or parts thereof.

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22. The pharmaceutical composition of claim 21, wherein the agent is a cytokine.

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23. The pharmaceutical composition of claim 22, wherein the cytokine is selected from the group consisting of GDF, GDNF, TGF, activin A, BMP, BDNF, NGF, EGF, CNTF and FGF.

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24. A diagnostic kit comprising the nucleic acid according to anyone of claims 1 to 7 and/or the vector of claim 8 and/or the protein of claim 10 and/or the antibody of claim 11 for the detection of neurodegenerative disorders and/or infections of the CNS in mammals.

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25. The diagnostic kit of claim 24, wherein the infection is a meningitis.

26. The diagnostic kit of claim 24 or 25, wherein the mammal is a human.

Abstract

The present invention relates to a transforming growth factor-
5 beta (TGF- β)-like protein which is derived from neurons and glial
cells, and which has a neurotrophic effect on dopaminergic
(DAergic) neurons, to nucleic acids coding for the protein, to a
vector containing the nucleic acids, to host organisms containing
the nucleic acids or the vector, to antibodies directed against the
10 protein, to methods for the production of the nucleic acids, the
vector or the protein, to a pharmaceutical composition for the
treatment of neurodegenerative disorders in mammals and to a
diagnostic kit for the detection of said disorders.